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Applicant: HemeBiotech A/S
Roskildevej 12 C
3400 Hillerød
DK

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Karin Schlichting
Head Clerk

FIELD OF THE INVENTION

The present invention relates to novel methods of treating and preventing disease caused by absence or deficiency of the activity of enzymes belonging to the heme biosynthetic pathway. More specifically, the invention pertains to methods of alleviating the symptoms of certain porphyrias, notably acute intermittent porphyria.

BACKGROUND OF THE INVENTION

Heme biosynthetic pathway

Heme is a vital molecule for life in all living higher animal species. Heme is involved in such important processes as oxygen transportation (haemoglobin), drug detoxification (cytochrome P450), and electron transfer for the generation of chemical energy (ATP) during oxidative phosphorylation in mitochondria.

Heme is synthesised in eight consecutive enzymatic steps starting with glycin and succinyl-CoA. Sassa S. 1996, Blood Review, 10, 53-58 shows a schematic drawing of the heme biosynthetic pathway indicating that that the first enzymatic step (ALA-synthetase) and the last three steps (coproporphyrinogen oxidase, protoporphyrinogen oxidase and ferrochelatase) are located in the mitochondrion whereas, the remaining are cytosolic enzymes.

Important regulation of the heme biosynthetic pathway is delivered by the end product of the metabolic pathway, namely heme, which exerts a negative inhibition on the first rate-limiting enzymatic step (conducted by ALA-synthetase) in the heme biosynthetic pathway. (Strand et al. Proc. Natl. Acad. Sci. 1970, 67, 1315-1320).

Deficiencies in the heme biosynthetic enzymes have been reported leading to a group of diseases collectively called porphyrias.

A defect in the third enzymatic step leads to acute intermittent porphyria, AIP.

Acute Intermittent Porphyria

Acute intermittent porphyria (AIP) is an autosomal dominant disorder in man caused by a defect (50% reduction of activity) of the third enzyme in the heme biosynthetic pathway, porphobilinogen deaminase, (also known as porphobilinogen ammonia-lyase(polymerizing)), E.C. 4.3.1.8.(Waldenström J. Acta.Med. Scand. 1937 Suppl.82.). In the following, this enzyme will be termed "PBGD".

Clinical manifestation of AIP

The reduction in enzymatic PBGD activity makes this enzyme the rate limiting step in the heme biosynthetic pathway, with a concomitant increase in urinary and serum levels of delta-aminolevulinic acid (ALA) and porphobilonogen (PBG).

The clinical manifestation of AIP involves abdominal pain and a variety of neuropsychiatric and circulatory dysfunctions. As a result of the enzymatic block, heme precursors such as porphobilinogen (PBG) and delta-aminolevulinic acid (ALA) are excreted in excess amounts in the urine and stool. In acute attacks, high levels of PBG and ALA are also found in serum. These precursors are normally undetectable in serum in healthy individuals.

The neuropsychiatric disturbances observed in these patients are thought to be due to interference of the precursors with the nervous system or due to the lack of heme. For instance, ALA bears a close resemblance to the inhibitory neurotransmitter 4-aminobutyric acid (GABA) and has been suggested to be a neurotoxin. (Jeans J. et al. American J. of Medical Genetics 1996,65, 269-273).

Abdominal pain is the most frequent symptom in AIP patients and occurs in more than 90% during acute attacks, which will be followed rapidly by the development of peripheral neuropathy with weakness in proximal muscles, loss of pinprick sensation, and paraesthesia. Tachycardia, obstipation or diarrhoea may also be present.

During acute attacks behavioural changes, confusion, seizures, respiratory paralysis, coma and hallucinations may be present.

Hypertension is also associated with AIP, with as high as 40% of patients showing sustained hypertension between attacks. An association between chronic renal failure (Yeung L. et al. 1983, Q J. Med 52, 92-98) and AIP as well as hepatocellular carcinoma. (Lithner F. et al. 1984, Acta.Med.Scand. 215,271-274.), has been reported.

The AIP is a lifelong disease which usually becomes manifest in puberty.

Factors precipitating acute attacks.

Most precipitating factors exhibit an association with the first rate-limiting enzyme in the heme biosynthetic pathway through heme, the final product of the pathway. A lowering of the heme concentration will immediately increase the rate of ALA-synthetase. An overproduction of ALA then makes the partially deficient PBGD enzyme (50% activity) now rate-limiting with an accumulation of the heme precursors ALA and PBG. Drugs that induces cytochrome P450 such as barbiturates, estrogens, sulphonamides, progesterone, carbamyazepine, and phenytoin can all precipitate acute attacks. (Wetterberg L. 1976, In Doss M. Nowrocki P. eds. Porphyrias in Human Disease. Reports of the discussion. Matgurg an der Lahn, 191-202).

The clinical manifestation is more common in women, especially at time of menstruation. Endocrine factors such as synthetic estrogens and progesterone are known precipitating factors. A significant factor is also the lack of sufficient caloric intake. Hence, caloric supplementation during acute attacks reduces clinical symptoms. (Welland F.H. et al. 1964, Metabolism,13, 232).

Finally, various forms of stress including illness, infections, surgery and alcoholic excess have been shown to lead to precipitation of acute attacks. There are also cases of acute attacks where no precipitating factor can be identified.

Prevalence of AIP

Prevalence of 0.21% has been reported (Tishler P.V: et al. 1985, Am.J.Psychiatry 142,1430-1436), with as high a prevalence as 1 per 1500 in geographic isolates in northern Sweden

(Wetterberg L. 1967, Svenska bokförlaget Nordstedt,Stockholm). Prevalence up to 200 per 10.000 inhabitants have been reported from northern Finland. (Andersson, Christer, Thesis, 1997, ISBN 91/7191/280/0).

Existing treatment of AIP

The treatment of AIP as well as of other types of porphyrias such as variegata, hereditary coproporphyria, harderoporphyria, and aminolevulinic acid dehydratase deficiency, are basically the same. Existing therapies for AIP, are all aimed at reducing circulating PBG and ALA by inhibiting the first rate-limiting enzymatic step ALA-synthetase. This inhibition of ALA-synthetase is achieved by increasing circulating heme, since heme is a negative feed back regulator of ALA-synthetase. Hematin treatment, high caloric intake or inhibition of heme breakdown by Sn-mesoporphyrin administration are the existing therapies today. These therapies have shown limited efficacy.

Treatment between acute attacks involves sufficient caloric intake and avoidance of drugs and immediate treatment of infections.

Patients that experience acute attacks are treated with intravenous carbohydrates usually dextrose (300 g/day) and intravenous hematin (3 - 8 mg/(kg day)).

Treatments with long acting agonistic analogues of LHRH, have been shown to reduce the incidence of pre-menstrual attacks by inhibiting ovulation in AIP patients. Finally, treatments involving heme analogues Sn-mesoporphyrin, which inhibit heme breakdown have also been attempted.

Medical need in AIP

The lack of effective treatment for AIP is well recognised. In a US mortality study in AIP patients requiring hospitalisation it was concluded that the mortality rate was 3.2-fold higher as compared to a matched general population.
Suicide was also a major cause of death, occurring at a rate of 370 times that expected in the general population (Jeans J. et al.1996, Am. J. of Medical Genetics 65, 269-273).

Hematin therapy is usually initiated when high caloric intake is not sufficient to alleviate acute attacks. Studies with hematin have been performed but these studies generally used the patients as their own control after the patients did not respond to high carbohydrate treatment. (Mustajoki et al. 1989, Sem. Hematol. 26, 1-9).

The one controlled study with hematin treatment reported, failed to reach statistical significance due to too small a patient number (Herrick A.L. et al 1989, Lancet 1, 1295-1297).

In conclusion, there is a definite need for the provision of novel therapeutic/prophylactic methods aimed at these diseases.

DISCLOSURE OF THE INVENTION

Levels of ALA and PBG found in urine in patients with symptomatic AIP, are in the range of 1-203 mg/day and 4-782 mg/day, respectively. Normal excretion of ALA and PBG is very low (0-4 mg/day). Important is the observation that these patients also have elevated levels of ALA and PBG in serum. It was shown in a study that AIP patients had significantly elevated levels of ALA (96 µg %) and PBG (334 µg %) in serum in connection with acute attacks and that the severity of the attacks were correlated to high levels of ALA and PBG. Hence, it is important to reduce the circulating levels of ALA and PBG in order to eliminate clinical symptoms and to normalize the heme pool.

The present inventors present a new therapeutic rational in the treatment of AIP, a rationale using PBGD, preferably recombinant PBGD (rPBGD) in order to reduce circulating high levels of PBG in serum by metabolising (by enzymatic conversion) PBG to hydroxymethylbilane (HMB), which is the normal product of the reaction. This substitution therapy will lead to a normalization of PBG in serum as well as to a normalization of the heme pool. It will also lead to a normalization of ALA in serum, since these heme precursors are in equilibrium with each other. A lowering of serum ALA and PBG is expected to result in a concomitant relief of symptoms. The product of the reaction (HMB) will diffuse back into the cells and enter the normal heme biosynthetic pathway and will become subsequently metabolized to heme.

Hence, PBGD administered by injections will carry out its normal catalytic function by converting PBG to HMB in serum (extracellularly, not inside the cells), where it normally functions. The new therapeutic idea is based on the assumption that ALA, PBG and HMB permeate cellular membranes or are transported specifically across them. An alternative to this is to administer a form of PBGD which will be able to act intracellularly, either as a consequence of formulation or as consequence of modification of PBGD so as to facilitate its entry into cells from the extracellular compartment.

The observation that AIP patients have large amounts of these heme precursors in the serum supports the idea that PBG does not accumulate intracellularly, but is released from the cells into serum when the intracellular concentration increases due to the PBGD enzymatic block.

The basic new therapeutic concept for AIP is valid for all porphyrias and therefore the invention is in general aimed at treating these diseases by substituting the reduced or missing enzymatic activity characterizing the porphyrias.

Hence, in its broadest aspect, the invention pertains to a method for treatment or prophylaxis of disease caused by deficiency, in a subject, of an enzyme belonging to the heme biosynthetic pathway, the method comprising administering, to the subject, an effective amount of a catalyst which is said enzyme or an enzymatically equivalent part or analogue thereof.

Hence, by the term "catalyst" is herein meant either the relevant enzyme which is substituted as it is, or an enzymatically equivalent part or analogue thereof. One example of an enzymatically equivalent part of the enzyme could be a domain or subsequence of the enzyme which includes the necessary catalytic site to enable the domain or subsequence to exert substantially the same enzymatic activity as the full-length enzyme.

An example of an enzymatically equivalent analogue of the enzyme could be a fusion protein which includes the catalytic site of the enzyme in a functional form, but it can also be a homologous variant of the enzyme derived from another species. Also, completely synthetic molecules which mimic the specific enzymatic activity of the relevant enzyme would also constitute "enzymatic equivalent analogues".

In essence, the inventive concept is based on the novel idea of substituting the reduced enzymatic activity in the subject simply by administering a catalyst which will "assist" the enzyme which is in deficit. The precise nature, however, of the catalyst is not all-important. What is important is merely that the catalyst can mimic the enzymatic *in vivo* activity of the enzyme.

Previous investigations in treating the porphyrias have suggested gene therapy, thus aiming at introducing genetic material in relevant cells, which will then take over the *in vivo* production of the enzyme of interest. The present approach is much more simple but has to the best of the knowledge of the inventors never been suggested, perhaps because difficulties with respect to achieving an intracellular effect on the precursor level has been regarded as an obstacle.

The term "the heme biosynthetic pathway" refers to the well-known enzymatic steps (cf. e.g. Sassa S. 1996, Blood Review, 10, 53-58) which leads from glycin and succinyl-CoA to heme, and enzymes belonging to this synthetic pathway are porphobilinogen deaminase (PBGD), ALA dehydratase, Uroporphyrinogendecarboxylase, Coproporphyrinogen oxidase, Coproporphyrinogen oxidise, Protoporphyrinogen oxidase, Uroporphyrinogen III synthase, Ferrochelatase, and Uroporphyrinogen decarboxylase. Hence, in line with the above, a catalyst used according to the invention is such an enzyme or an enzymatically equivalent part or analogue thereof. It should be noted that all of the above-mentioned enzymes have been sequenced, thus allowing recombinant or synthetic production thereof.

The diseases related to reduced activity of these enzymes are acute intermittent porphyria (AIP), ALA deficiency porphyria (ADP), Porphyria cutanea tarda (PCT), Hereditary coproporphyria (HCP), Harderoporphyria (HDP), Variegata porphyria (VP), Congenital erythropoetic porphyria (CEP), Erythropoietic protoporphyria (EPP), and Hepatoerythropoietic porphyria (HEP).

By the term "effective amount" is herein meant a dosage of the catalyst which will supplement the lack of enzymatic activity in a subject suffering from porphyria caused by reduced activity of one of the above-mentioned enzymes. The precise dosage constituting an effective amount will depend on a number of factors such as serum half-life of the catalyst, specific activity of the catalyst etc. but the skilled person will be able to determine the correct dosage in a given case

by means of standard methods (for instance starting out with experiments in a suitable animal model so as to determine the correlation between blood concentration and enzymatic activity).

The disease which is the preferred target for the inventive method is AIP, and therefore the catalyst is PBGD or an enzymatically equivalent part or analogue thereof. It is most preferred that the catalyst is a recombinant form of the enzyme belonging to the heme biosynthetic pathway or of the enzymatically equivalent part or analogue thereof, since recombinant production will allow large-scale production which, with the present means available, does not seem feasible if the enzyme would have to be purified from a native source.

Preferred formulations and dosage forms of the catalyst are exemplified for, but not limited to, PBGD in the detailed description hereinafter, and these formulations also are apparent from the claims. It will be appreciated that these formulations and dosage forms are applicable for all catalysts used according to the invention.

One important embodiment of the method of the inventions is one wherein the catalyst, upon administration, exerts at least part of its enzymatic activity in the intracellular compartment. This can e.g. be achieved when the catalyst is an enzymatically equivalent part or analogue of the enzyme, since such variations of the enzyme can be tailored to render them permeate cell membranes. Hence, when the catalyst is a small artificial enzyme or an organic catalyst which can polymerize porphobilinogen to hydroxymethylbilane, it should be possible for the skilled man to introduce relevant side chains which facilitates entry into the intracellular compartment. Alternatively, the catalyst is the enzyme, but formulated in such a manner that it exerts at least part of its enzymatic activity intracellularly upon administration to the subject. This can be achieved by tagging the enzyme with specific carbohydrates or other liver cell specific structures for specific liver uptake, *i.e.* the enzyme (or analogue) is modified so as to facilitate active transport into e.g. liver cells.

Although the above embodiments are interesting, it is believed that the normal, practical embodiment of the invention will involve use of a catalyst which exerts substantially all its enzymatic activity extracellularly in the bloodstream, since it is believed that the metabolic products of the enzymatic conversion of the relevant heme precursor will permeate freely into the intracellular compartment where the remaining conversions of the heme biosynthetic

pathway can take place. Alternatively, the metabolic product may be excreted from the subject via urine and/or faeces at least to some extent.

As mentioned above, it is preferred that the catalyst is produced recombinantly, *i.e.* by a method comprising

a) introducing, into a suitable vector, a nucleic acid fragment which includes a nucleic acid sequence encoding the catalyst;
b) transforming a compatible host cell with the vector;
c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence; and
d) recovering the expression product from the culture
and optionally subjecting the expression product to post-translational processing, such as in vitro protein refolding, enzymatic removal of fusion partners, alkylation of amino acid residues, and deglycosylation, so as to obtain the catalyst.

For relatively small catalysts (e.g. those constituted mainly of the active site of the enzyme), the catalyst can alternatively be prepared by liquid-phase or solid-phase peptide synthesis.

A more detailed explanation of the recombinant production of the model enzyme PBGD is given in the detailed section hereinafter, but as mentioned herein the same considerations apply for all other peptide catalysts of the invention. One of the main advantages of producing the catalyst by recombinant or synthetic means is, that if produced in a non-human cell, the catalyst is free from any other biological material of human origin, thus reducing problems with antigenicity etc.

The dosage regimen will normally be comprised of at least one daily dose of the catalyst,(preferably by the intravenous route). Normally 2, 3, 4 or 5 daily dosages will be necessary, but if sustained release compositions are employed, fewer than 1 daily dosage are anticipated.

The daily dosage should be determined on a case by case basis by the skilled practitioner, but as a general rule, the daily dosage will be in the range between 0.01 – 1.0 mg/kg body weight per day of the catalyst. More often the dosage will be in the range of 0.05 – 0.5 mg/kg body weight

per day, but it should never be forgotten that precise dosage depends on the dosage form and on the activity of the catalyst as well as on the degree of deficiency of the relevant enzyme.

For PBGD, the daily dosage is about 0.08-0.2 mg per kg body weight per day, and most often 0.1 mg per kg body weight per day will be the dosage of choice. It is believed that comparable dosages will be applicable for the other full-length enzymes.

Finally, as will be appreciated from the above disclosure, the invention is based on the novel idea of providing substitution for the enzymes lacking in activity. To the best of the knowledge of the inventors, therapeutic use of catalysts having such effects have never been suggested before, and therefore the invention also pertains to a catalyst as defined herein for use as a pharmaceutical. Furthermore, use of such catalysts for the preparation of pharmaceutical compositions for treatment of the above-discussed diseases is also part of the invention.

DETAILED DISCLOSURE OF THE INVENTION

The following description of preferred embodiments of the invention will focus on recombinant production of PBGD and formulations and uses thereof. It will be appreciated, however, that all disclosures relating to this polypeptide apply also for the other enzymes mentioned above. Hence, production and use of PBGD only exemplifies the invention, but all other enzymes of the heme biosynthetic pathway can be substitute PBGD in the embodiments described hereinafter.

Production of recombinant PBGD

As mentioned above, it is preferred to administer recombinant versions of the various enzymes of the heme biosynthetic pathway. In the following will be described recombinant production of one of these enzymes, namely PBGD.

The gene for the erythropoietic PBGD, which is located in the human genome in the chromosomal region 11q 24, is composed of 15 exons spanning 10 kb of DNA and is shown in Grandschamp B. et al. 1996. J. of Gastroenerology and Hepatology 11, 1046-1052.

The gene coding the erythropoietic PBGD enzyme (344 amino acids) (Raich N. et al 1986, Nucleic. Acid. Res, 14, 5955-5968), will be cloned from a human erythropoietic tissue by reverse transcriptase or amplification by PCR (polymerase chain reaction) of the PBGD coding region.

The gene will be inserted in a plasmid (bacterial: B-G&F-PBGD, yeast: Y-G&F-PBGD, or mammalian: M-G&F-PBGD) and transformed into a suitable host cell (a bacterium such as E. coli and B. subtilis, a fungus such as Saccharomyces, or a mammalian cell line, such as CHO cells). The expression of the PBGD gene will be regulated by a promoter which is compatible with the selected host cell.

For production in E. coli the PBGD coding sequence can be inserted into a plasmid vector, e.g. pBR322 or PUC.

For yeast production, the PBGD coding sequence can be inserted into a plasmid vector, for example YEP type, containing 2 u origin for high expression in yeast. YEP plasmids contain TRP 1 and URA 3 as markers for selective maintenance in trp1-, ura 3-yeast strains.

Alternatively, the PBGD gene can be inserted in bovine papilloma virus vectors BPV for high expression in a murine cell line C-127(Stephens P.E. et. al. Biochem J. 248, 1-11, 1987) or vectors compatible with expression in CHO cells or COS cells.

For bacterial production: Insert in front of the PBGD coding region a bacterial signal sequence for example an E. coli periplasmic enzyme signal peptide or a signal peptide from a secreted enterotoxin or endotoxin in E. coli, to obtain secretion in E. coli. An ATG sequence at the NH2-terminal end of the rPBGD structural gene for initiation of translation or a short leader sequence with an ATG at the 5´-end for cytoplasmic expression.

A secretory signal in Saccharomyces, for example alpha-mating factor presequence, can be added in front of the rPBGD structural gene for efficient secretion in yeast.

Similarly, a sequence encoding a mammalian signal peptide can be added for secretion of rPBGD into the culture medium upon expression in for example CHO cells or COS cells.

A bacterial promoter for example the tryptophane (trp) promoter or the lac promoter or alternatively an alkaline phosphatase promoter, should be inserted before the PBGD coding region for efficient transcription in prokaryotes for example E. coli .

A yeast promoter for example 3-phosphoglycerate kinase (PGK) or chelatin or alpha-mating factor should be inserted before the PBGD coding region for efficient transcription in yeast for example Saccharomyces cerevesiae or Saccharomyces pombe.

A mammalian promoter for example Metallothionin-1 (MT-1) or Aspartame transcarbamylase or Dihydrofolate reductase (DHFR) should be inserted before the PBGD coding region for efficient transcription in mammalian cell lines for example CHO cells or COS cells.

The bacterial plasmid (B-G&F-PBGD) containing a bacterial promoter, signal peptide or only an ATG sequence in front of the PBGD coding region, and the PBGD coding region and vector sequences from for example pBR322, will be transformed into a bacterial host strain for example E. coli for production of rPBGD.

The yeast plasmid (Y-G&F-PBGD) containing a yeast promoter, signal and/or ATG codon (methionine) in front of the PBGD coding region and a yeast vector containing selectable markers such URA 3 or TRP 1 will be transformed into the yeast host cell such as Saccharomyces cerevesiae or Saccharomyces pombe for production of rPBGD.

The mammalian plasmid (M-G&F-PBGD) containing a mammalian promoter for example Metallothionine-1 or Dihydrofolate reductase and a mammalian signal sequence or an ATG codon in front of the PBGD coding region and vector pAT or pSV2 respectively. Plasmid (M-G&F PBGD) will be transfected into a mammalian cell line for example CHO cells, for production of rPBGD.

The E. coli cell containing plasmid (B-G&F-PBGD), will be fermented to stationary phase between 24-48 hours, in a medium containing casein hydrolysate, or yeast extract, glucose, vitamins and salts. pH oxygen will be monitored by electrodes during fermentation. Temperature will be kept at 37 +/- 2 C during the fermentation.

The yeast cell containing the plasmid (Y-G&F-PBGD), will be fermented to late log phase between 20-40 hours in a medium containing yeast extract, glucose, salts and vitamins. pH and temperature will be monitored by specific electrodes during fermentation. Temperature will be kept at 30 +/- 2 C during fermentation.

The mammalian cell line containing the plasmid (M-G&F-PBGD) will be fermented in a medium containing, foetal calf serum (or serum free), vitamins, glucose, antibiotics, growth factors. pH and temperature will be monitored continuously during fermentation by specific electrodes.

The PBGD enzyme will be purified from the cells after fermentation.

rPBGD will recovered from E. coli after fermentation by an extraction procedure involving for example ribipress, sonication, osmotic shock or total solubilization by detergent for example Tween 80, Triton X-100 or Brij. rPBGD will be recovered from fermentation medium after production in yeast or from a total cellular extract using detergents such as Triton X-100, Tween 80 or Brij. rPBGD will be recovered from mammalian culture medium or from a total cellular extract by ion-exchange chromatography or affinity chromatography.

rPBGD will be purified from E. coli extract or from yeast medium or total cellular extract or from mammalian culture medium or total mammalian cellular extract by binding to an ion-exchange column for example DEAE-Sepharose or MonoQ-Sepharose and eluted with for example NaCl and Sodium phosphate buffer pH 7-8 or the corresponding potassium salts.
Alternatively, rPBGD will be recovered from extracts by binding to an affinity chromatography column for example an anti-PBGD affinity column. rPBGD will be eluted by lowering the pH to 4-2, or a thiol specific affinity column. rPBGD has been "tagged" with thiols residues when a thiol affinity column step is used. Thiols will be removed by a specific enzymatic cleavage step to generate authentic rPBGD.

The ion-exchange or affinity purified rPBGD will be further purified by hydrophobic interaction chromatography on for example, TSK Phenyl 5 PW column or Octyl-Sepharose or Phenyl-Sepharose columns.

Binding of rPBGD will be done at high ionic strength for example in 10-50 mM Tris-HCl pH 7-8, 1M NaCl or 10-15 mM Sodium phosphate pH 7-8, 0.5 M $MgSO_4$ and eluted by lowering the ionic strength for example with 10-50 mM Tris-HCl pH 7-8 or 10-50 mM Sodium phosphate pH 7-8.

Three hydrophobic interaction steps will be applied consecutively.

rPBGD will be further purified with preparative RP-HPLC for example C12 or C18 matrixes. The rPBGD will be eluted from the column by a gradient of 10-50 mM Sodium phosphate and 1-10% acetonitrile buffer.

Formulation of rPBGD will be done by passing the enzyme over a G-100 Sephadex column and eluting it in an isotonic solution for example 0.9%NaCl and 10-50mM Sodium phosphate pH7.0 +/- 0.5 or Sodium phosphate, glycin, mannitol or the corresponding potassium salts.

The formulation solution of rPBGD will be sterile filtered and filled aseptically in glass vials and lyophilised.
Alternatively, the sterile filtered rPBGD solution will be formulated in for example, lipid vesicles constituting phosphatidylcholine or phosphatidylethanolamine or combinations of these or incorporated into erythrocyte ghosts.

Reconstitution of lyophilised rPBGD will be done in water for injection.

Alternatively, rPBGD will be formulated in a sustained release formulation involving a biodegradable microspheres, for example in polylactic acid., polyglycolic acid or mixtures of these.

Alternatively, rPBGD will be lyophilized in a two-compartment cartridge, where rPBGD will be in the front compartment and water for reconstitution in the rear compartment. This two compartment cartridge will be combined with an injection device to administer either rPBGD by a needle or needle less (by high pressure) device.

Alternatively, rPBGD will be formulated in a physiological buffer containing an enhancer for nasal administration.

Alternatively, rPBGD will be formulated in an oral formulation containing for example, lipid vesicles (phospatidylcholine, phosphatidylethanolamine, sphingomyeline) or dextrane microspheres.

Although recombinant production of PBGD is preferred for the treatment of AIP, it can alternatively be produced from human red blood cells.

The production and manufacturing of recombinant PBGD will be done by the following steps.

RECOMBINANT PBGD PRODUCTION PROCESS; AN OUTLINE

A: Fermentation

1. Master cell bank
2. Working cell bank
3. Production of seed culture
4. Fermentation in large fermenter (250 L >)

B. Purification

1. Cell concentration by filtration/centrifugation
2. Cell disruption
3. Ultrafiltration
4. Chromatography ion exchange, DEAE-Sepharose, MonoQ-Sepharose
5. Hydrophobic interaction chromatography (Octyl/phenyl-Sepharose, TSK Phenyl, 5PW, Phenyl - Sepharose
6. Chromatography ion exchange (MonoQ)
7. Formulation by Gel filtration Sephadex G-100

C. Manufacturing

1. Sterile filtration
2. Aseptic filling

3. Lyophilization

TREATMENT OF OTHER PHORPHYRIAS

In analogy with the new treatment of AIP patients with (recombinant) PBGD, hepatic Porphyrias such as ALA dehydratase ALA deficiency Porphyria (ADP), Porphyria- cutanea tarda (PCT), Hereditary Coproporphyria (HCP) and Variegata Porphyria (VP) can benefit from substitution therapy by rALA dehydratase, rUroporphyrinogen decarboxylase, rCoproporphyrinogen oxidase and rProtoporphyrinogen oxidase, respectively.

Patients having Erythropoetic Porphyrias such as Congenital erythropoietic Porphyria (CEP) or Erythropoietic protoporphyria (EPP) will benefit from substitution therapy with rUroporphyrinogen III syntetase and rFerrochelatase, respectively.

Hepatoerythropoietic Porphyrias e.g.. Hepatoerythropoietic Porphyrias(HEP) can be treated with rUroporphyrinogen decarboxylase.

All porphyrias can be treated by the administration of the enzymatic activity lacking or being reduced (normally 50%) in any of the eight steps in the heme biosynthetic pathway as described above.

The substitution of the enzymatic activity can be achieved by adding the corresponding recombinant enzyme or other molecules that will provide the missing enzymatic activity.

SEQUENCING OF PBGD

Introduction:

The goal of this segment of the project is to clone and sequence the erythropoietic expressed form of porphobilinogen deaminase (PBGD)(Raich et. al. 1986.) Two forms of PBGD are known. The erythropoietic form is expressed specifically in erythroid progenitors and the constitutive form is expressed in all cells (Grandchamp et. al. 1987.) The two are expressed from the same gene and are identical except for the

addition of 17 amino acids at the amino terminus of the constitutive form through alternative exon usage. It was decided to clone and express the erythropoietic form. There are three sequences for PBGD in the Genebank, the two isoforms mentioned above and the genomic sequence (Yoo et. al. 1993.) These all have nucleotide differences translating to amino acid changes. Before choosing a specific sequence to be expressed for a human therapeutic it was therefore necessary to determine what is the wild type allele. To accomplish this, PBGD cDNA clones were isolated and sequenced from a number of sources to define the most common amino acid usage. Oligonucleotide primers were designed to amplify the coding region from cDNAs by Polymerase Chain Reaction (PCR)(Saiki et. al. 1985.) These were used to isolate cDNAs from 5 sources of mRNA which were then cloned into a plasmid vector. Eight of these clones were sequenced and along with the published sequences define a wild type allele which should be the most common amino acid sequence in the population. This wild type allele will be used for protein expression.

Strategy:

A nested PCR strategy was devised to clone PBGD. The first primer set, lco379 and lco382, are 20mers that bind to sequence outside of the coding region. lco379 is specific for the 5′ untranslated region of the mRNA (cDNA) of the erythropoietic form of PBGD. The binding site is in an exon region not expressed in the constitutive form of the enzyme. lco382 binds to the 3′ untranslated region of both forms of PBGD. Internal to these are a second set of oligonucleotide primers to be used for the second round of PCR, lco375 and lco376, designed to distal ends of the PBGD coding region. lco375 has 22 bases of sequence homologous to the 5′ end of the coding region of the erythropoietic form of PBGD with the ATG start codon followed by an EcoRI endonuclease cleavage site for cloning of the PCR product and 4 bases of sequence to ensure efficient restriction. lco376 has 33 bases homologous to the 3′ end of the PBGD coding region with 3 bases changed internally to introduce a MunI/MfeI endonuclease cleavage site through silent mutations and ending with the TAA stop codon. This restriction site will be used to easily introduce sequence encoding a His-Tag to the DNA with oligonucleotide adapters or to enable other 3′ modifications. Following the stop codon is a second stop codon to ensure good

termination of translation and a HindIII endonuclease cleavage site for cloning the PCR product followed by 4 bases to ensure efficient restriction. The EcoRI and HindIII endonuclease cleavage sites introduced onto the ends of the PBGD PCR product ligate into the respective unique restriction sites in the high copy number pBluescriptII SK- (Stratagene) vector for sequencing and will then be used to move the PBGD DNA into an *E. coli* expression vector for production of recombinant human porphobilinogen deaminase, rh-PBGD.

PCR:

Six cDNAs were used as a PCR source; spleen, bone marrow, lymph node, lung, whole brain and adipose tissue each from a different pool of human donors (produced by Donald Rao using BRL Superscript II with 500 ng Clontech poly-A RNA in 20 μl reaction volumes per manufacturers instructions except adipose which was made from 5 μg of Clontech total RNA from a single donor.). One μl of each cDNA (approximately 25ng) was amplified with Advantage cDNA polymerase mix (Clontech) with 0.2mM dNTP (PE/ABI) and 0.3μM each of lco379 and lco382 in 50 μl reaction volumes. Two cycle PCR was used, with an initial heat denaturation step at 94°C for 1' 40" then 28 cycles of 96°C for 16" and 68°C for 2'. A final extension of 6' at 74°C ensured that extension products were filled out. One fifth of the reaction was run out on a 1.2% agarose gel with 2 μl of 6X ficol loading dye in 0.5X TBE buffer (Maniatis, 1982.) The predicted band of 1.1 kb. was observed by ethidium bromide staining with all sources but lung tissue cDNA. These bands were excised and DNA was isolated with Microcon-30 with micropure inserts (Amicon/Millipore) per manufacturers instructions and buffer exchanged with dH2O. One tenth of the recovered DNA was amplified with Advantage cDNA polymerase mix (Clontech) with 0.2mM dNTP (PE/ABI) and 0.3μM each of the internal nested oligonucleotides (lco375 and lco376) at 0.3μM in 50μl reactions. Two cycle PCR was used again with an initial heat denaturation step at 94°C for 1' 40" then 2 cycles of 96 C for 16" and 68°C for 2' then

13 cycles of 96°C for 16" and 72°C for 2' with a final extension of 6' at 74°C. Ten µl of the 50 µl reactions were run on a 1.2% agarose gel with 2 µl 6X loading dye. The resulting bands were of the expected size, 1.05 kb. The remainder of the PCR reactions were passed through Chromaspin-400 columns (Clontech) per manufacturers instructions to remove reaction components and to exchange buffer with TE (10mM Tris-HCl pH8.0/ 1mM EDTA). The DNA containing eluates were washed with dH2O and concentrated with Microcon-100 spin-filters (Amicon/Millipore) per manufacturers instructions.

Cloning:

The purified PBGD DNA was digested for 6 hours with 40 Units each of EcoRI and HindIII in EcoRI "U" buffer (New England Biolabs (NEB)) in 50 µl reactions at 37°C. Enzymes were heat killed for 20 minutes at 68°C and reactions were spun in Microcon 100 spin-filters to remove small DNA end pieces, washed with dH2O and concentrated. One fifth of the resulting DNA was ligated with approximately 50 ng EcoRI and HindIII digested and twice gel purified pBluescriptII SK- (Stratagene) and 200 units T4 DNA ligase (NEB cohesive end units) for 15 hours at 16°C. The ligase was heat killed at 75°C for 10 minutes. The reactions were then buffer exchanged with dH2O and concentrated in Microcon-100 spin filters and volumes taken up to 5 µl with dH2O. One µl each was electroporated into 25 µl DH10B Electromax cells (Gibco/BRL) at 2.5Kv/200Ohms/25µF in 0.1cm cuvets with a BioRad electroporator. One ml of SOC medium (Gibco/BRL) was added and the cells were outgrown at 37°C for one hour at 250 rpm. Cells were plated out on LB plates (Maniatis, 1982) with 150 µg/ml ampicillin. The efficiency of all five were approximately twice background (vector ligated without insert.) Colony PCR was used to analyze 18 transformants of each electroporation for the presence of PBGD. An internal PBGD specific primer (lco381) was used with a

pBluescript specific primer (Ico385) to both confirm identity and proper orientation in the vector. The 25 μl reactions were set up on ice to inactivate proteases with primer concentrations of 0.4μM, 0.125U Taq polymerase (Fisher), and 0.2mM dNTP(PE/ABI.) Two cycle PCR was used, with an initial heat denaturation step at 94°C for 1' 40" a further denaturing step at 96°C for 20 seconds, then 30 cycles of 96°C for 16" and 68°C for 1' with a final extension of 4' at 74°C. Five μl of 6X loading dye was added and 12.5 μl each were run out on a 1.2% agarose gel. Results are as follows: 12/18 positive colonies for spleen; 10/18 for bone marrow; 8/18 for lymph node; 9/18 for brain and 10/18 for adipose tissue. Two positive colonies each for the first 3 and 1 each for the latter two were grown up in 25 ml. liquid LB culture with 150 μg/ml ampicillin over night at 37°C with 250 rpm. Plasmid DNA was purified from the cultures with Qiagen's Tip-100 DNA purification kit per manufacturer's instructions. UV absorbance at 260nm was used to determine the plasmid yields which varied from between 131 and 169 μg of highly purified DNA.

Sequencing:

Sequencing reactions of double stranded plasmid DNA with Big Dye terminator cycle sequencing were performed in a 9700 thermocycler (Perkin Elmer/Applied Biosystems.) Two vector primers (Ico383 and Ico385) and two PBGD specific internal primers (Ico380 and Ico381) were used for all 8 plasmids (see sequence). In addition a fifth vector primer (Ico285) was used for the brain and adipose derived clones. Reaction conditions were per manufacturers protocol as follows: 500 ng plasmid DNA and 4 pmol oligonucleotide primer with 8 μl ready mix in 20 μl volumes with 30 cycles of 96°C for 12" and 60°C for 4'. Extension products were purified by isopropanol precipitation. To each reaction 20 μl of dH2O and 60 μl Isopropanol were added. These were mixed by inversion and allowed to sit at

room temperature for 15 minutes then spun for 40' at 3250 rpm in a Beckman GS-6KR centrifuge with the GH3 rotor and Microplate + carriers. Reactions were inverted then spun at 1680 rpm for 1' to remove liquid from the pelleted DNA. DNA sequence analysis was performed at the University of Washington Biochemistry Department sequencing Laboratory with an Applied Biosystems 377 sequencer.

Analysis:

The inserts of all 8 clones were confirmed to be PBGD by complete double strand sequence analysis (see sequence alignment.) Each has some change(s) from the published sequences. Some changes are unique and some are shared with other clones (see Figure 1.) For differences found only in one clone, it is difficult to distinguish between PCR or cloning artifacts and actual allelic variations without additional sampling. However, when the same base difference is found in more than one sequence it is unlikely to be from cloning errors. From the alignment of all 11 PBGD sequences a set of common bases emerged, the consensus or wild type allele sequence. Five of the eight clones (1.1, 1.3, 2.1, 3.3, and 5.3.) have the wild type amino acid sequence. Within this set with wild type amino acid sequence, there is only one difference at the nucleic acid level. At position 555, 4 of the 5 sequences have a dGTP while 1 along with the published erythropoietic and genomic PBGD have a dTTP. These appear to be two common alleles which result in no amino acid difference. There are 2 base changes between clone number 1.1 and the published erythropoietic PBGD. An adenine to guanine change at base 513 (Leu 171) is a silent mutation which is also present in 9 out of the 11 sequences compared. The second difference is a cytosine to adenine substitution at base 995 (Thr 332.) This is not a silent change, with a threonine to asparagine non-conservative mutation. It appears however that the difference is an error in the published erythropoietic PBGD sequence since

all 10 other sequences have an adenine at this position. In addition to these natural variations, there are three additional silent mutations introduced during the cloning at positions 1017, 1018 and 1020 to create a Mun-I site for future manipulations.

Conclusion:

For any recombinant therapeutic protein it is important that the wild type allele be used to reduce the potential for immunogenicity. We feel confident through our survey of the literature and analysis of PBGD sequence from different individuals that clone number 1.1 represents the most prevalent "wild type" allele in the population with respect to amino acid sequence. Clone number 1.1 contains the consensus wild type amino acid sequence and differs from the published erythropoietic PBGD sequence by only one amino acid. Because this difference is found in all the other PBGD clones besides the erythropoietic PBGD sequence, it, rather than the published erythropoietic sequence, is deamed to be the prevalent wild type sequence. For this reason PBGD encoded by clone number 1.1 was chosen for production of recombinant human porphobilinogen deaminase (rh-PBGD.)

Table of oligonucleotide primers:

lco375-pbgds (32 mer) coding region 5' end w/ EcoRI site sense
5' CGT GGA ATT CAT GAG AGT GAT TCG CGT GGG TA 3'

lco376-pbgda (47 mer) coding region 3' end w/ HindIII site antisense
5' GGA GAA GCT TAT TAA TGG GCA TCG TTC AAT TGC CGT GCA ACA TCC AG 3'

lco378-csnonc (20 mer) constitutive form non-coding sense
5' TCC AAG CGG AGC CAT GTC TG 3'

lco379-esnonc (20 mer) erythropoietic form non-coding sense
5' TCG CCT CCC TCT AGT CTC TG 3'

lco380-sinter (21 mer) internal coding sense
5' CAG CAG GAG TTC AGT GCC ATC 3'

lco381-ainter (21 mer) internal coding antisense
5' GAT GGC ACT GAA CTC CTG CTG 3'

lco382-anonc (20 mer) non-coding sense
5' CAG CAA CCC AGG CAT CTG TG 3'

lco383-pSKT7 (22 mer) pBluescript T7 promoter
5' GTA ATA CGA CTC ACT ATA GGG C 3'

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lco384- pSKpjrev (22 mer) pBluescript reverse1
5' CTA AAG GGA ACA AAA GCT GGA G 3'

lco385- pSKrev (21 mer) pBluescript reverse2
5' CAG CTA TGA CCA TGA TTA CGC 3'

Equipment list:

Item	Manufacturer	Serial Number
Pipetman P-1000	Gilson	N55287E
Pipetman P-200	Gilson	N52324E
Pipetman P-20	Gilson	N53465M
Pipetman P-10	Gilson	P626586
5415C centrifuge	Eppendorf	5415B68381
GS-6KR centrifuge	Beckman	NGD97J18
Avanti J-25 I centrifuge	Beckman	JJY97J14
DU 640B Spectrophotometer	Beckman	4323015
Genie II vortex	VWR	2-241186
GeneAmp PCR system 2400	Perkin Elmer (PE) / Applied Biosystems (ABI)	803N6021903
GeneAmp PCR system 2400	PE / ABI	803S7100104
GeneAmp PCR system 9700	PE / ABI	805S7121566
1545 incubator	VWR	0902597
heat block 1	VWR	0795
heat block 1	VWR	0511
Gene Pulser II Apparatus	BioRad	340BR2745
Pulse Controller Plus	BioRad	339BR1377
Power Pac 1000	BioRad	286BR00770
Sub Cell	BioRad	16S/8860
Wide-Mini Sub Cell	BioRad	02S/7951
Foto/Prep transilluminator	Fotodyne	PTG1-0997-2831
Elutrap Electro-separator	Schleicher + Schuell	Order No. 57880
Innova 4000 incubator	New Brunswick Scientific	890165366
Power Mac G3 computer	Macintosh	XA8061A3BBW
Trinitron Multiscan 200GS monitor	Sony	8057052
DNA analysis Software: Geneworks	Intelligenetics	Version 2.5.1

Supplies List:

Item	Supplier	Cat No.	Lot No.
Human Spleen Poly A+ RNA	Clontech	6542-1	7120266
Human Bone Marrow Poly A+ RNA	Clontech	6573-1	56714
Human Lung Poly A+ RNA	Clontech	6524-1	7050104
Human Lymph Node Poly A+ RNA	Clontech	6594-1	6120292
Human Brain Poly A+ RNA	Clontech	6516-1	63101
Human Adipose Total RNA	Clontech	D6005-01	7907005
Superscript II reverse transcriptase	Gibco/BRL	18064-014	JM6418
100 mM dNTP set	Pharmacia	27-2035-01	6072035011
pBluescriptII SK- phagemid	Stratagene	212206	0270702
Advantage cDNA polymerase mix	Clontech	8417-1	8060354
GeneAmp dNTP	PE/ABI	N-808-0007	H0172..4,H0553
Xba-I endonuclease	New England Biolabs (NEB)	145S	30
Pvu-II endonuclease	NEB	151L	14
EcoR-I endonuclease	NEB	101L	25
Hind-III endonuclease	NEB	104S	49
Tris six-Pack "C"	Sigma	T-PAC-C	77H9049
0.5 M EDTA pH 8.0	Sigma	E-7889	16H8924
Chromaspin TE 400	Clontech	K1323-1	7090795
Chromaspin 400 DepC dH2O	Clontech	K1333-1	7040086
Quiaquick gel extraction kit	Qiagen	28704	BY97017/0397/119

25

Microcon-30	Amicon	42410	L8JM4330B
Microcon-100	Amicon	42413	L8DM3296A
Micropure 0.22μm	Amicon	42544	CCB017
Seakem GTG agarose	FMC	50074	709397
100 bp DNA Ladder	NEB	323-1	3
123 bp DNA Ladder	Gibco/BRL	15613-029	JK9706
T4 DNA Ligase	NEB	202S	64
Ampicillin	Sigma	A-9518	76H0434
LB media	Gibco/BRL	12795-084	12E1072B
Bacto Agar	Difco	0140-07-4	106728JA
DH10B electromax	Gibco/BRL	18290-015	KHN430
SOC media	Gibco/BRL	15544-042	1010351
Taq polymerase	Fisher	FB-6000-15	H0436
TaqStart antibody	Clontech	5400-1	6070479
Qiafilter Midi DNA isolation kit	Qiagen	12243	PO No. 514
Isopropanol	Sigma	I-9516	47H3724
Big Dye terminator cycle sequencing kit	PE/ABI	4303152	9803008

Variation of PBGD clones from published erythroid sequence:

PBGD clone	Differences from Erythroid mRNA: silent	non-silent	total diffs	Genebank No.	Reference/Source
Erythroid	0	0	0	X04217	Raich,N. et. Al. Nucleic Acids Res. 14 (15), 5955-5968 (1986)
Constitutive	1	2	3	X04808	Grandchamp,B. et. Al. Eur. J. Biochem. 162 (1), 105-110 (1987)
Genomic	1	2	3	M95623	Yoo,H.W. et. Al. Genomics 15 (1), 21-29 (1993)
1.1	1	1	2	-	Spleen (Clontech mRNA Lot No. 7120266)
1.3	2	1	3	-	Spleen (Clontech mRNA)
2.1	2	1	3	-	Bone Marrow (Clontech mRNA)
2.2	2	2	4	-	Bone Marrow (Clontech mRNA)
3.1	2	4	6	-	Lymph Node (Clontech mRNA)
3.3	3	1	4	-	Lymph Node (Clontech mRNA)
5.3	2	1	3	-	Total Brain (Clontech mRNA)
6.1	3	2	5	-	Adipose Tissue (Clontech mRNA)

Figure 2:

Summary of the number of differences in amino acid sequence of our sequenced PBGD clones and clones from Genebank entrys for the constitutive and genomic PBGD with published Erythropoietic PBGD sequence. Shown in different columns are the total number of silent mutations with a DNA base change not causing a corresponding amino acid change, the number of non-silent mutations with a DNA change causing an amino acid difference and the sum of the two types of mutations. Not included in this figure are the three silent mutations introduced into the clones to create an internal Mun-I endonuclease cleavage site. Note that clone number 1.1 which will be used for

production of recombinant human porphobilinogen deaminase (rh-PBGD) has only one of each type of difference with the least number of total differences.

Summary of mutations found in PBGD clones:

aa.	aa No.	bp No.	mutation	aa change	cons.	gen.	1.1	1.3	2.1	2.2	3.1	3.3	5.3	6.1	No. /10
Asp	19	56	A -> G	Asp -> Gly							X				1
Phe	108	322	T deletion	frame shift						X					1
Lys	140	419	A -> G	Lys -> Arg							X				1
Leu	160	478	C -> A	Leu -> Met		X									1
Ala	168	503	C -> T	Ala -> Val							X				1
Leu	171	513	A -> G	silent		X	X	X	X	X	X	X	X	X	9
Val	185	555	T -> G	silent	X			X	X	X		X	X	X	7
Glu	193	577	G -> A	Glu -> Lys	X										1
Gly	243	729	C -> T	silent								X			1
Ala	280	840	T -> C	silent										X	1
Ala	286	856	G -> A	Ala -> Thr										X	1
Lys	328	984	A -> G	silent						X					1
Thr	332	996	C -> A	Thr -> Asn	X	X	X	X	X	X	X	X	X	X	10
Gln	339	1017	G -> A	silent			X	X	X	X	X	X	X	X	8
Gln	339	1018	C -> T	silent			X	X	X	X	X	X	X	X	8
Leu	340	1020	T -> G	silent			X	X	X	X	X	X		X	7
Leu	340	1020	T deletion	frame shift									X		1

Figure 1:

Summary of the genetic differences of our sequenced PBGD clones and Genebank entrys for the constitutive and genomic PBGD with published erythropoietic PBGD sequence from the allele sequence alignment. Listed in different columns are the amino acid, base number from the ATG start codon, the actual genetic difference with corresponding amino acid change if any and a listing of the clones with differences shown with an X. The final column tallys the number of clones with that mutation. The final four mutations are introduced with Ico376 during PCR amplification to create a Mun-I endonuclease cleavage site. Note that clone number 1.1 which will be used for production of rh-PBGD only has differences which are represented by a number of clones.

References:

Grandchamp B, et al Tissue-specific expression of porphobilinogen deaminase. Two isoenzymes from a single gene. Eur J Biochem. 1987 Jan;162(1):105-10.

Maniatis T., E.F. Fritsch, J. Sambrook. Molecular Cloning (A laboratory Manual) Cold Spring Harbor Laboratory. 1982.

Raich N, et al Molecular cloning and complete primary sequence of erythrocyte porphobilinogen deaminase. Nucleic Acids Research 1986 14(15) :5955-67

Saiki RK, et al Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985 Dec 20;230(4732):1350-4.

Yoo HW, et. Al. Hydroxymethylbilane synthase: complete genomic sequence and amplifiable polymorphisms in the human gene. Genomics 1993 Jan; 15(1):21-9.

CLAIMS

1. A method for treatment or prophylaxis of disease caused by deficiency, in a subject, of an enzyme belonging to the heme biosynthetic pathway, the method comprising administering, to the subject, an effective amount of a catalyst which is said enzyme or an enzymatically equivalent part or analogue thereof.

2. A method according to claim 1, wherein the disease is selected from the group consisting of
acute intermittent porphyria (AIP),
ALA deficiency porphyria (ADP),
Porphyria cutanea tarda (PCT),
Hereditary coproporphyria (HCP),
Harderoporphyria (HDP),
Variegata porphyria (VP),
Congenital erythropoetic porphyria (CEP),
Erythropoietic protoporphyria (EPP), and
Hepatoerythropoietic porphyria (HEP).

3. A method according to claim 1 or 2, wherein the catalyst is an enzyme selected from the group consisting of
porphobilinogen deaminase (PBGD)
ALA dehydratase,
Uroporphyrinogendecarboxylase,
Coproporphyrinogen oxidase,
Coproporphyrinogen oxidise,
Protoporphyrinogen oxidase,
Uroporphyrinogen III synthase,
Ferrochelatase, and
Uroporphyrinogen decarboxylase,
or an enzymatically equivalent part or analogue thereof.

4. A method according to any of the preceding claims, wherein the disease is AIP and the enzyme is PBGD or an enzymatically equivalent part or analogue thereof.

5. A method according to any of the preceding claims, wherein the catalyst is a recombinant form of the enzyme belonging to the heme biosynthetic pathway or of the enzymatically equivalent part or analogue thereof.

6. A method according to any of the preceding claims, wherein the catalyst is administered by a route selected from the group consisting of the intravenous route, the intraarterial route, the intracutaneous route, the subcutaneous route, the oral route, the buccal route, the intramuscular route, the anal route, the transdermic route, the intradermal route, and the intratechal route.

7. A method according to any of claims 1-6, wherein the catalyst is formulated in an isotonic solution, such as 0.9% NaCl and 10-50 mM Sodium phosphate pH 7.0 +/- 0.5 or Sodium phosphate, glycine, mannitol or the corresponding potassium salts.

8. A method according to any of claims 1-7, wherein the catalyst is lyophilised.

9. A method according to any of claims 1-8, wherein the catalyst is sterile filtered.

10. A method according to any of claims 1-6, 8 or 9, wherein the catalyst is formulated as lipid vesicles comprising phosphatidylcholine or phosphatidylethanolamine or combinations thereof

11. A method according to any of claims 1-6, 8 or 9, wherein the catalyst is incorporated into erythrocyte ghosts.

12. A method according to any of claims 1-6, 8 or 9, wherein the catalyst is formulated as a sustained release formulation involving biodegradable microspheres, such as microspheres comprising polylactic acid, polyglycolic acid or mixtures of these.

13. A method according to any of claims 1-9, wherein the catalyst is lyophilized in a two-compartment cartridge, where the catalyst will be in the front compartment and water for reconstitution in the rear compartment.

14. A method according to claim 13, wherein the two compartment cartridge is combined with an injection device to administer the catalyst either by a needle or by a needle-less (high pressure) device.

15. A method according to any of claims 1-9, wherein the catalyst is formulated in a physiological buffer containing an enhancer for nasal administration.

16. A method according to any of the preceding claims, wherein the catalyst is formulated as an oral formulation containing lipid vesicles, such as those comprising phospatidylcholine, phosphatidylethanolamine, or sphingomyeline, or dextrane microspheres.

17. A method according to any of the preceding claims, wherein the catalyst is formulated so as to enhance the half-life thereof in the subject's bloodstream.

18. A method according to claim 17, wherein the catalyst has a polyethylene glycol coating.

19. A method according to claim 17, wherein the catalyst is complexed with a heavy metal.

20. A method according to any of the preceding claims, wherein the catalyst is an enzymatically equivalent part or analogue of the enzyme and exerts at least part of its enzymatic activity intracellularly upon administration to the subject.

21. A method according to claim 20, wherein the catalyst is a small artificial enzyme or an organic catalyst which can polymerize porphobilinogen to hydroxymethylbilane

22. A method according to any of claims 1-9, wherein the catalyst is said enzyme formulated in such a manner that it exerts at least part of its enzymatic activity intracellularly upon administration to the subject.

23. A method according to claim 22, wherein the catalyst is tagged with specific carbohydrates or other liver cell specific structures for specific liver uptake.

24. A method according to claims 1-19, wherein the catalyst exerts substantially all its enzymatic activity extracellularly in the bloodstream.

25. A method according to claim 24, wherein the enzymatic activity of the catalyst on its relevant heme precursor results in a metabolic product which 1) either moves into the intracellular compartment and is converted further via the remaining steps of the heme biosynthetic pathway or 2) is excreted from the subject via urine and/or faeces.

26. A method according to any of the preceding claims, wherein the catalyst has been prepared by a method comprising

a) introducing, into a suitable vector, a nucleic acid fragment which includes a nucleic acid sequence encoding the catalyst;
b) transforming a compatible host cell with the vector;
c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence; and
d) recovering the expression product from the culture
and optionally subjecting the expression product to post-translational processing, such as in vitro protein refolding, enzymatic removal of fusion partners, alkylation of amino acid residues, and deglycosylation, so as to obtain the catalyst.

27. A method according to any of claims 1-25, wherein the catalyst has been prepared by liquid-phase or solid-phase peptide synthesis.

28. A method according to any of the preceding claims, wherein the catalyst is free from any other biological material of human origin.

29. A method according to any of the preceding claims, wherein the catalyst is administered at least once a day, such as 2, 3, 4, and 5 times daily.

30. A method according to any of the preceding claims wherein the daily dosage is in the range of 0.01 – 1.0 mg/kg body weight per day, such as in the range of 0.05 – 0.5 mg/kg body weight per day.

31. A method according to any of the preceding claims, wherein the daily dosage is about 0.1 mg per kg body weight per day.

32. A catalyst which is an enzyme of the heme biosynthetic pathway or an enzymatically equivalent part or analogue thereof, for use as a medicament.

33. Use of a catalyst which is an enzyme of the heme biosynthetic pathway or an enzymatically equivalent part or analogue thereof for the preparation of a pharmaceutical composition for the treatment or prophylaxis of diseases caused by deficiency of said enzyme.

34. The use according to claim 33, wherein the treatment or prophylaxis is performed according to any of claims 1-31.

35. A method according to any of claims 1-31 wherein the catalyst is a recombinant form of the enzyme according to any of sec. no. 1 to 3 or an analogue thereof.

36. A catalyst according to claim 32, which is recombinant human PBCD based on the clone 1.1 as described herein.

SEQUENCE LISTING

<110> Gellerfors, Par
Fogh, Jens

<120> New therapeutic method for treating patients with Acute Intermittent Porphyria (AIP) and other porphyric diseases

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